Insect Biochemistry and Molecular Biology 43 (2013) 474–482

Contents lists available at SciVerse ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

# Bacterial infection activates the immune system response and dysregulates microRNA expression in honey bees

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ARTICLE INFO

*Article history:*
Received 30 November 2012
Received in revised form
16 February 2013
Accepted 4 March 2013

*Keywords:*
*Apis mellifera*
Antimicrobial peptides
KappaB sites
Transcriptional regulation
miRNA

ABSTRACT

In insects, a rapid and massive synthesis of antimicrobial peptides (AMPs) is activated through signaling pathways (Toll and Imd) to combat invading microbial pathogens. However, it is still unclear whether different types of bacteria provoke specific responses. Immune response mechanisms and the activation of specific genes were investigated by challenging *Apis mellifera* workers with the Gram-negative bacterium *Serratia marcescens* or the Gram-positive bacterium *Micrococcus luteus*. The immune system responded by activating most genes of the Toll and Imd pathways, particularly AMP genes. However, genes specifically regulated by *M. luteus* or *S. marcescens* were not detected, suggesting an interaction between the signaling pathways that lead to immune effectors synthesis. Despite this finding, kappaB motifs in the 5′-UTRs of selected genes suggest a pathway-specific control of AMP and *transferrin-1* gene expression. Regulation by miRNAs was also investigated and revealed a number of candidates for the post-transcriptional regulation of immune genes in bees.



## 1. Introduction

The innate immune system of insects comprises an array of cellular and humoral components that function to eliminate invading parasites and pathogens. Cellular immunity includes phagocytosis, nodule formation, and encapsulation mediated by hemocytes, whereas humoral defense refers to soluble molecules, such as antimicrobial peptides (AMPs), complement-like proteins, and enzymatic cascades, that regulate melanin formation and clotting (Strand, 2008). The first step in the immune response requires the recognition of pathogens by molecules, such as peptidoglycan recognition proteins (PGRPs) and Gram-negative bacteria-binding proteins (GNBPs). Once these components are activated, the Toll and Imd signaling cascades are triggered, ultimately resulting in the induction of AMPs by NF-kappaB factors (Lemaitre and Hoffmann, 2007).

In *Drosophila*, specific recognition proteins are activated by the appropriate pathogen-associated molecular pattern to initiate signaling cascades. Surface molecules present on Gram-negative bacteria are recognized by receptors in the Imd pathway, leading to the nuclear translocation of the NF-kappaB transcription factor Relish and the induction of specific AMPs. In contrast, challenges with fungi or Gram-positive bacteria activate the Toll pathway, resulting in the translocation of Dif/Dorsal (NF-kappaB factors) into the nucleus and the synthesis of distinct AMPs (Lemaitre and Hoffmann, 2007).

The screening of the published honey bee genome (Honey Bee Genome Sequencing Consortium, 2006) allowed for the prediction of immune components of pathways implicated in inducible host defense (Evans et al., 2006). Although the number of immune gene families was fewer relative to other insects, all gene categories, from recognition and signaling to immune effectors, were predicted in honey bees (Evans et al., 2006). However, only a few expression studies of immune-related genes focusing on the dynamics of signaling pathway activation and its transcriptional regulation in bees have been conducted so far.

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http://dx.doi.org/10.1016/j.ibmb.2013.03.001

MicroRNAs (miRNAs) are important post-transcriptional regulators of gene expression that have been implicated in fine-tuning a large variety of biological processes. Recent studies in mammals have demonstrated that miRNAs are essential in both adaptive and innate immunity, including the control of differentiation of various immune cell subsets as well as their immunological functions (O'Connell et al., 2007; Lu and Liston, 2009). The involvement of miRNAs in the immune system has also been described in insect species (Winter et al., 2007; Garbuzov and Tatar, 2010; Skalsky et al., 2010; Freitak et al., 2012; Fullaondo and Lee, 2012). Post-transcriptional regulation of the immune system by miRNAs may also occur in the honey bee, but as far as we know, the role of miRNAs in bee immunity has not yet been studied.

Here, we compared the immune responses of young honey bee workers that were experimentally infected with two different bacterial species, and bees that had been injured, using quantitative RT-qPCR to contrast the expression of nine immune-related genes that are representative of the two main signaling pathways (Toll and Imd) and iron sequestration defense (transferrin). The use of two types of bacteria for adult honey bee infection, Gram-positive *Micrococcus luteus* and Gram-negative *Serratia marcescens*, allowed us to investigate whether the preferential activation of one of the two pathways occurred in response to a particular type of bacteria. The potential regulatory elements of NF-kappaB/Rel family factors were also investigated in the 5′-upstream regions of the selected genes. Additionally, we examined potential miRNAs as post-transcriptional regulators of immune genes in honey bees.

## 2. Materials and methods

### 2.1. Insects

Africanized *Apis mellifera* were taken from hives of the Experimental Apiary of the Department of Genetics, Faculty of Medicine, University of São Paulo, Ribeirão Preto, Brazil. All experiments were conducted with adult workers; newly emerged bees (0–16 h old) were collected and separated into groups for subsequent treatment.

### 2.2. Bacterial inoculation

Groups of 15–20 newly emerged bees were injected in the dorsal side between the 5th and 6th abdominal segment with 1 μL of *M. luteus* (bacteria in log phase; $OD_{600} = 1$, diluted 28× in nuclease-free water), 1 μL of *S. marcescens* (bacteria in log phase; $OD_{600} = 1$, diluted 20× in nuclease-free water) or 1 μL of nuclease-free water. The injections were performed using a Hamilton micro syringe (1701LT) with a G30 needle (Becton & Dickinson). Then, the bees were maintained at 34 °C and 80% RH and fed a diet consisting of 30% beebread (pollen processed by bees and stored in the hive) and 70% sugar. Water was given *ad libitum* to all groups. Eight to twelve bees were randomly collected at different time points following treatment (0.5, 1, 3, 6, and 12 h for bees injected with *S. marcescens* and 3 and 6 h for bees injected with *M. luteus* or water). The controls were non-injected bees collected at time zero.

For miRNA analysis, a new experiment was carried out in which the bees were injected with *S. marcescens* as described above, and the controls (non-injected bees) were taken at the same time point as the experimental group (6 h post-treatment).

### 2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from the abdominal carcass (integument and adhered fat body) using 1 mL of TRIzol® reagent (Invitrogen). Each sample was prepared with abdomens dissected from 2 to 4 bees. RNA samples were treated with RNase-free DNase (Promega) to eliminate contaminant genomic DNA.

For mRNA quantification, cDNA was synthesized using 3 μg of total RNA, Superscript II (Invitrogen) and oligo $(dT)_{12-18}$ (Invitrogen). For the detection and quantification of miRNAs, cDNA was synthesized from total RNA (1.5 μg) using the NCode™ First-strand cDNA Synthesis kit (Invitrogen) following the manufacturer's protocol.

### 2.4. Quantification of mRNA expression by quantitative and semi-quantitative RT-PCR

Transcripts of selected genes that are known to be part of the Toll and Imd pathways (*dorsal-1B*, *PGRP-S3*, *B-glucan 2*, *relish*, and *cactus-2*) as well as immune effector genes in honey bees (*abaecin*, *hymenoptaecin*, *defensin-1*, and *transferrin-1*) (Evans et al., 2006) were quantified using RT-qPCR. The expression levels of these genes were analyzed using a 7500 Real-Time PCR System (Applied Biosystems). Amplification was carried out in a 20 μL reaction volume containing 10 μL SYBR® Green Master Mix 2× (Applied Biosystems), 1 μL cDNA (diluted 10×), 7.4 μL water and 0.8 μL (8 pmol) of each gene-specific primer (Supplementary material Table S1). The PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. To check reproducibility, the SYBR® Green assay was performed in duplicate or triplicate. Quantitative measurements were normalized using *A. mellifera rp49* mRNA levels as internal control; this gene and *actin* are suitable for gene expression data normalization in honey bees (Lourenço et al., 2008). The specificity of the PCR products was verified by melting curve analysis for all samples. Data were analyzed according to the comparative threshold cycle (Ct) method, in which the amount of a target transcript, normalized to an endogenous reference and relative to a calibrator is indicated by $2^{-\Delta\Delta Ct}$. Ct indicates the PCR cycle number at which the amount of amplified target gene reached a fixed threshold. The ΔCt value was then determined by subtracting the average target gene Ct value from the average reference gene Ct value. The ΔΔCt value was determined by subtracting each ΔCt value from the calibrator ΔCt value, defined as one of the samples of the control group (non-injected bees).

To evaluate the temporal pattern of AMP gene activation, semi-quantitative analyses were performed using the following temperature profile: 94 °C (2 min) and 25–27 cycles of 94 °C (30 s), 60 °C (30 s) and 72 °C (30 s), with a final extension step at 72 °C (10 min). PCR products were analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized by ultraviolet light. The intensity of each band signal was quantified by densitometry using the Kodak 1D Image Analysis program. Quantitative measurements were normalized using *A. mellifera actin* mRNA levels as an internal control. The relative intensity of the bands for each sample was calculated by dividing the intensity of the target cDNA band by the intensity of *actin* cDNA band from the corresponding sample.

### 2.5. Quantification of mature miRNAs by RT-qPCR

Amplification reactions (using the 7500 Real-Time PCR System) were performed for each miRNA using miRNA-specific primers and the NCode Universal qPCR reverse primer (Invitrogen). The forward primer sequences for each miRNA corresponded to the mature *A. mellifera* miRNA sequences available at miRBase (http://www.mirbase.org). Amplification was carried out in a 20 μL reaction volume containing 10 μL SYBR® Green Master Mix 2× (Applied Biosystems), 2 μL cDNA (diluted 10×), 7.2 μL water and 0.4 μL (4 pmol) of each primer (forward/reverse). The PCR conditions were 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s